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TITLE: Controlling Mitochondrial Dynamics to Mitigate Noise-Induced Hearing Loss

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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14. ABSTRACT

In this study, we are examining the efficacy of a potential early intervention therapeutic for noise induced hearing loss (NIHL). The generation of reactive oxygen species is one of the underlying mechanisms of noise-induced damage to tissues in the inner ear that leads to noise-induced hearing loss (NIHL). The goal is to define an effective post-exposure, non-invasive intervention strategy to mitigate a primary cause of loud-sound induced hearing loss: mitochondrial dysfunction and overproduction of reactive oxygen species. Utilizing a localized application procedure to the outer ear canal of a small molecule mitochondrial fission inhibitor, mitochondrial division inhibitor-1, the results so far have demonstrated that inhibition of the mitochondrial fission process significantly attenuates NIHL and reduces damage to the tissues of the inner ear.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION

The generation of reactive oxygen species (ROS) is one of the underlying mechanisms of noise-induced damage to tissues in the inner ear that leads to noise-induced hearing loss (NIHL). Following loud sound exposure, the generation of excessive ROS by mitochondria in many different tissues of the inner ear is well recognized. Mitochondrial dysfunction, including the deregulation of fission and fusion processes, is implicated in many human pathological conditions including hearing loss. The studies proposed here will test the novel hypothesis that inhibiting the mitochondrial fission process will mitigate the deleterious effects of loud sound on hearing sensitivity. In our preliminary studies, we discovered that application of mitochondrial division inhibitor-1 (mdivi-1) to the outer ear canal after loud sound exposure significantly reduced noise-induced auditory threshold shifts in our mouse model of NIHL. Additionally, protection against outer hair cell loss at the high frequency responsive region of the organ of Corti was observed. Importantly, these findings demonstrated that altering mitochondrial dynamics following noise exposure is a potential mechanism for intervention of NIHL. In this study, through a pharmacological approach, we are defining a post-exposure intervention strategy to mitigate a primary cause of loud-sound induced hearing loss: mitochondrial dysfunction and overproduction of reactive oxygen species.

2. KEYWORDS

Hearing loss, loud sound, mitochondria, reactive oxygen species, dynamin-related protein-1, mitochondrial division inhibitor-1,

3. ACCOMPLISHMENTS:

What were the major goals of the project?

A. Major Task 1: ACURO approval and equipment set-up. Dates: 09/15/2015 – 02/14/2015
B. Major Task 2: Identify effective dosing regimen for outer ear canal mdivi-1 application for a single loud sound exposure. Dates: 02/15/2016 – 08/14/2016
C. Major Task 3: Identify effective dosing regimen for intraperitoneal mdivi-1 injection. Dates: 08/15/2016 – 02/14/2016.
D. Major Task 4: Quantify cochlear mdivi-1 concentration. Dates: 02/15/2017 – 05/14/2017
E. Major Task 5: Identify molecular mechanisms affected by mdivi-1 treatment. Dates: 02/15/2017 – 03/14/2018.
F. Major Task 6: Identify effective dosing regimen for outer ear canal mdivi-1 application for multiple loud sound exposures. Dates: 02/15/2018 – 09/14/2018.

What was accomplished under these goals?

A. Major activities
   - Completing Major Task 1: ACURO approval and equipment purchase and set-up.
   - Performance of studies within Major Task 2.
   - Initiation of studies within Major Task 4.

B. Specific objectives

- Obtain ACURO approval.
- Complete the ordering and set-up of the ABR-DPOAE test systems and the sound exposure equipment and isolation booth.
- Continue testing mdivi-1 efficacy in reducing noise induced hearing loss through application to the outer ear canal (Major Task 2, Subtask 1). Analysis of low (50 uM) mdivi-1 dose, treatment to be performed both immediately following noise exposure and at 6 hours post-noise exposure.
- Perform analysis of outer auditory hair cells and synaptic ribbons from the different treatment groups (Major Task 2, Subtask 2).
- Set-up and standardization of a protocol for HPLC analysis of cochlear mdivi-1 concentrations in the inner ear (Major Task 4). This task is being completed with the assistance of the OHSU Bioanalytical Shared Resource/Pharmacokinetics Core.

C. Significant results or key outcomes

Major Task 1: ACURO approval and equipment set-up.

- The ABR-DPOAE test system set-up and calibration is completed and is currently in use for completion of the Tasks.
- Construction of the sound exposure booth and installation of fire sprinkler has been completed. Set-up and calibration of the sound exposure equipment will be completed by the end of October, 2016.

Major Task 2: Identify effective dosing regimen for outer ear canal mdivi-1 application for a single loud sound exposure.

Subtask 1: ABR/DPOAE measurements

We have made significant progress towards the completion of this Subtask that includes outer ear canal application testing of mdivi-1 (50 μM and 150 μM) treatment 1) immediately following noise exposure, and 2) at 6 hours post-noise exposure. The study groups are as follows: (1) mdivi-1/vehicle, (2) noise exposure + immediate mdivi-1/vehicle treatment, and (3) noise exposure + 6 hour post-noise exposure mdivi-1/vehicle treatment.

Methods for Subtask 1:

1. Loud sound exposure. Mice are put into compartments of a divided wire mesh cage and placed into the center of an open field acoustic chamber. Free access to food and water is provided. For these single loud sound exposure studies, a free field noise level of 103 dB SPL, 8-16 kHz sound with a 5 minute ramp up in noise levels is applied for 2 hours. Control animals are kept at ambient noise levels for an equivalent amount of time.

2. Outer ear canal application of mdivi-1. Mdivi-1 is dissolved in DMSO to a stock solution of 100 mM and further diluted in saline immediately prior to use (final DMSO is 0.23%). For

application of drug to the outer ear canal, the animal is lightly anesthetized with zylaxine (5 mg/kg)/ketamine (20 mg/kg) to prevent movement. Next, under a dissecting microscope, the animal is placed on their side with ear to be treated turned upwards, and 25 ul of the drug solution is applied to the outer ear canal using a pipette and sterile pipette tips. An equal volume of vehicle (saline + 0.23% DMSO) is applied to the contralateral outer ear canal. The dissecting microscope allows proper placement of the pipette tip at the opening of the outer ear canal (at a safe distance from the tympanic membrane) and visual confirmation that the solution has gone into the ear canal and is up against the tympanic membrane without the formation of bubbles.

3. Cochlear sensitivity measurements. ABR (auditory brainstem response) threshold levels and DPOAE (distortion otoacoustic emissions) levels were measured before each experiment to confirm normal auditory function as well as to assess noise-induced hearing threshold shifts. The animals were anesthetized with a mixture of xylazine (10 mg/kg, IP) and ketamine (40 mg/kg, IP) and placed on a heating pad in a sound-isolated chamber. The external ear canal and tympanic membrane was inspected using an operating microscope to ensure the ear canal was free of wax and that there was no canal deformity, no inflammation of the tympanic membrane, and no effusion in the middle ear. For the outer ear canal drug application studies, the individual performing the ABR/DPOAE measurements and analysis was blinded as to which ear of each animal had received the mdivi-1 versus saline treatment.

Auditory brainstem response: Needle electrodes are placed subcutaneously near the test ear, at the vertex and at the shoulder of the "test ear side." Each ear is stimulated separately with a closed tube sound delivery system sealed into the ear canal. The auditory brain-stem response to a 1-ms rise-time tone burst at 4, 8, 12, 16, 24, and 32 kHz is recorded and thresholds obtained for each ear. The intensity of tone burst stimulus is increased in steps of 5 dB. Threshold is defined as an evoked response of 0.2 μV from the electrodes.

Distortion product otoacoustic emissions: The "cubic" DPOAE at the frequency 2f1-f2 are generated by two tones played simultaneously to the ear. The stimuli consisted of two primary tones (f2/f1=1.2) at the level (L1=L2) 60 dB SPL that are emitted from speakers and presented over a range 4-32 kHz. The sound stimuli is generated by 24 bit 192 kHz ESI Wave terminal 192X Sound Card and an in house developed acoustic system. The DPOAE stimuli is delivered to the ear canal using a coupler tip fitted within the opening of the ear canal to form a closed acoustic system. The cubic distortion product is recorded in the ear canal by an Etymotic 10B microphone. The microphone is coupled to signal input channel of the sound card. For data analysis, the amplitude of the 2f1-f2 distortion product is plotted against the f2 frequency where a significant portion of the DP is generated.

Results of Subtask 1:

- Group (1) mdivi-1/vehicle. Treatment with mdivi-1 (50 μM) did not result in a significant change in ABR or DPOAE metrics (data not shown) (n=15 mice).
- Group (2) noise exposure + immediate mdivi-1/vehicle treatment. A significant reduction in hearing threshold shifts was observed at 24 kHz with mdivi-1 treatment (Figure 1)

(n=14 mice). This demonstrates that outer ear canal administration of mdivi-1 following noise exposure is effective in reducing loss of hearing sensitivity.

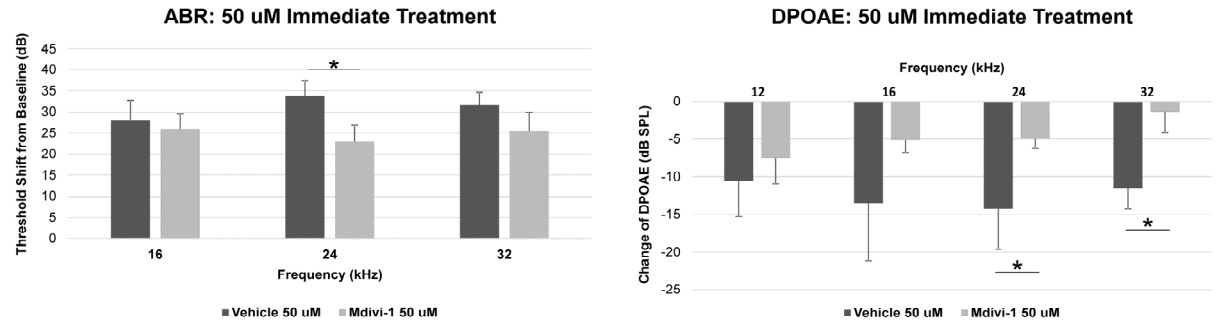


Figure 1: Mdivi-1 outer ear application immediately following loud sound exposure reduced loss of noise induced hearing sensitivity. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) metrics were measured prior to and again at 2 weeks post-noise exposure. Mice (n=14) were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours. Immediately following exposure, mdivi-1 (50 μM) was administered to one outer ear canal of each mouse. The contralateral ear received an equal volume of vehicle. Left panel: ABR analysis revealed a significant reduction in threshold shift at 24 kHz. *P<0.05. Right panel: Change of the cubic DPOAE (f1,f2=60 dB) with mdivi-1 treatment was also significant. Data are presented as mean+SEM.

- Group (3) noise exposure + 6 hour post-noise exposure mdivi-1/vehicle treatment. The preliminary results suggests that a small protective effect against loss of hearing sensitivity can be provided by mdivi-1 treatment at 6 hours post-noise exposure (n=6 mice). The reduction of ABR threshold shifts in the mdivi-1 treated group relative to vehicle did not reach statistical significance (P<0.07 at 24 kHz). However, the results

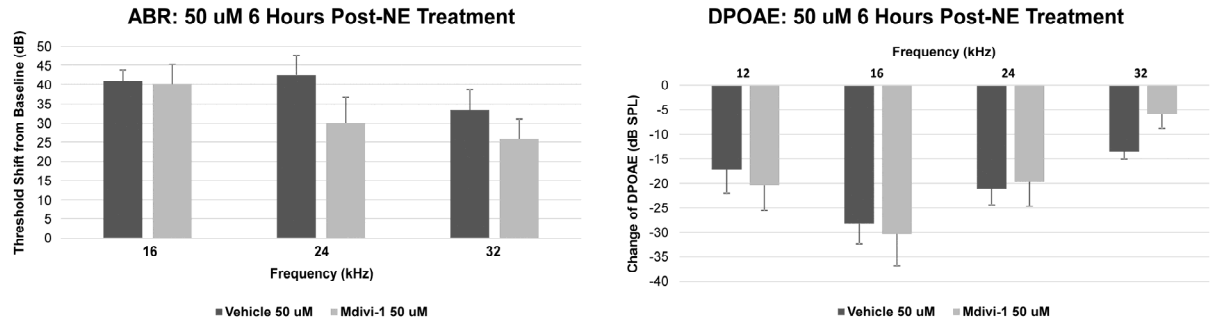


Figure 2: Mdivi-1 outer ear application at 6 hours following loud sound exposure shows a trend towards reduced loss of noise induced hearing sensitivity. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) metrics were measured prior to and again at 2 weeks post-noise exposure. Mice (n=6) were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours. At 6 hours following exposure, mdivi-1 (50 μM) was administered to one outer ear canal of each mouse. The contralateral ear received an equal volume of vehicle. Left panel: ABR analysis revealed reductions in threshold shifts. However, the P value did not reach significance (<0.07 at 24 kHz). Right panel: Change of the cubic DPOAE (f1,f2=60 dB) with mdivi-1 treatment did not demonstrate any attenuation of threshold shifts. Data are presented as mean+SEM.

presented are derived from a small group size (n=6), and this study is still in progress. Notwithstanding, the results do indicate that there is a "window of opportunity" following noise exposure in which the inhibition of mitochondrial fission will be effective in reducing noise-induced hearing loss. Treatment with the higher amount of mdivi-1 (150 μM) may prove effective.

Subtask 2: Histological analysis of OHC numbers.

We have made progress towards the completion of the outer hair cell counts (OHC) for this Subtask, particularly for study groups (1) mdivi-1/vehicle, and (2) noise exposure + immediate mdivi-1/vehicle treatment, with 50 μM mdivi-1. Synaptic ribbon images have been acquired for both groups and analysis is currently underway.

Methods for Subtask 2:

1. Auditory hair cell and synaptic ribbon counts: Following ABR/DPOAE measurements, the mice are deeply anesthetized with ketamine hydrochloride (100 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) and euthanized by decapitation. The cochleae are rapidly removed and perfused with 3.7% paraformaldehyde/0.25% glutaraldehyde in 0.1M phosphate buffer, fixed for 2.5 hours and decalcified overnight in Tris/10% EDTA. The organ of Corti is dissected into five sections and incubated overnight with anti-CtBP2 antibody to label synaptic ribbons. Following several PBS washes, the tissue sections are stained with Alexa Fluor 488 phalloidin and Hoechst 33258 to allow for visualization and counting of outer hair cells. Confocal images are obtained on an Olympus IX81 inverted microscope fitted with an Olympus Fluoview FV1000 confocal laser microscope system. Cytocochleograms of each dissected cochlea are generated and the number of hair cells present is counted and plotted as fractional survival relative to percent distance from the cochlea apex following standard protocol. Confocal z-stack projection are acquired at the 16, 32, and 64 kHz regions of the cochlea (identical regions of interest (ROI) are used consisting of 17 to 20 inner hair cells). The number of synaptic ribbons present per inner hair cell at each of the 3 frequencies is then counted.

Results of Subtask 2:

- Group (1) mdivi-1/vehicle. Examination of organ of Corti surface preparations at 2 weeks post-treatment revealed normal IHC and OHC morphology and complement for the low to high frequency regions in both the mdivi-1 (50 μM) and vehicle treated groups. Additionally, treatment with mdivi-1 did not result in a significant change in synaptic ribbon counts. We have analyzed 6 of the 15 mice from this groups and are still processing samples.
- Group (2) noise exposure + immediate mdivi-1/vehicle treatment. The sound exposure level used in this study results in significant outer hair cell loss at the very high frequency region of the cochlea (>45 kHz) and no loss of inner hair cells. In this Subtask, we observed that mdivi-1 treatment (50 μM) resulted in an approximately 25% reduction in OHC loss at the 55 kHz region of the cochlea (Figure 3). We have also analyzed 6 of the 14 mice from this group and are still processing samples.

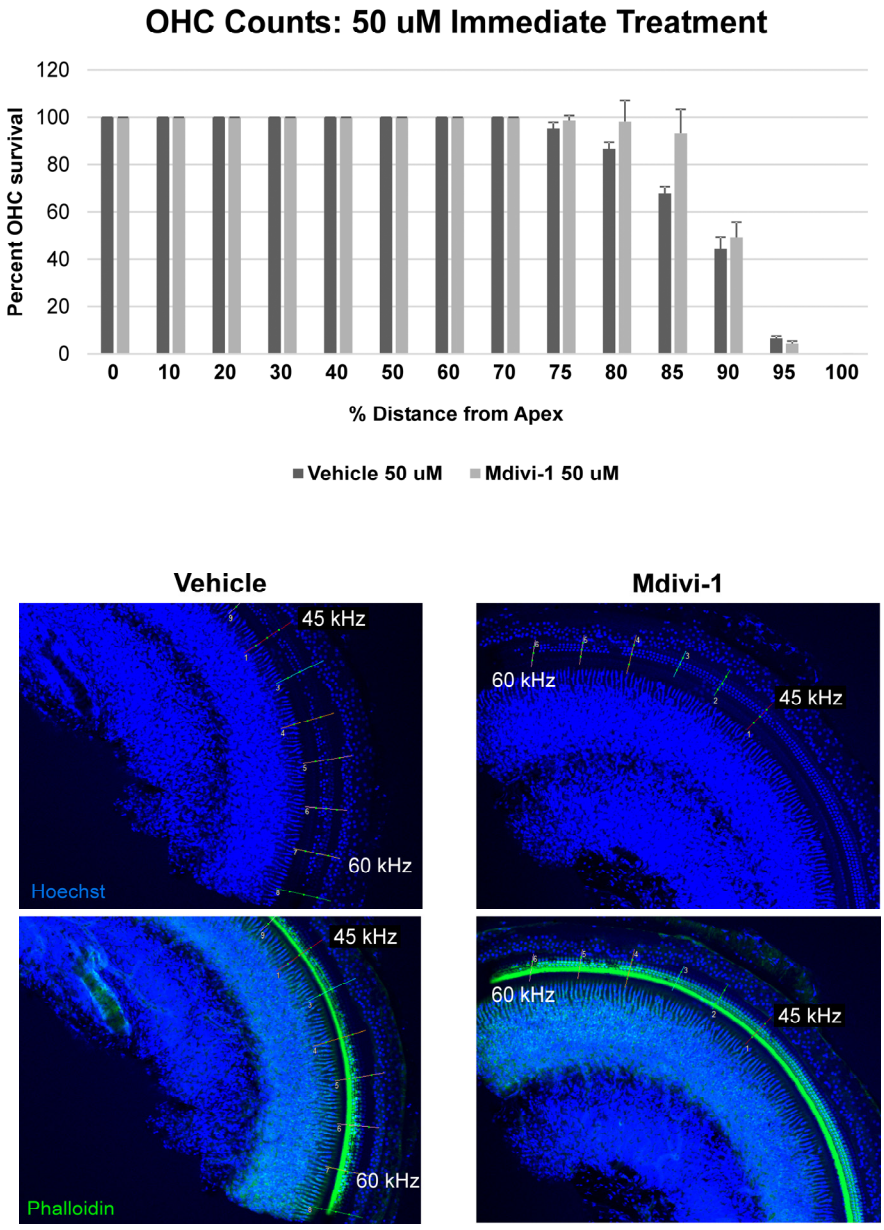


Figure 3. Mdivi-1 treatment reduces noise-induced loss of OHCs. Quantification of outer hair cell (OHC) loss was performed at 2 weeks post noise exposure after ABR and DPOAE measurements. Upper panel: Hair cell counts revealed significant reductions in OHC loss at the basal region of the basilar membrane with mdivi-1 treatment (n=6 cochlea) relative to vehicle treatment (n=6 cochlea). Lower panel: Representative organ of Corti surface preparation images of vehicle and mdivi-1 treated cochlea. Data are presented as mean+SEM.

D. Other achievements

None to report.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
We will continue to work toward completion of the goals of Major Task 2 which is the identification of the most effective dosing regimen for outer ear canal mdivi-1 application to mitigate loud sound induced hearing loss. This will involve completion of the low dose mdivi-1 treatment (50 uM) and analysis studies. We will begin the studies of the high dose mdivi-1 treatment (150 uM). For Major Task 4, quantification of cochlear mdivi-1 concentration, the set-up and standardization of HPLC analysis of cochlear mdivi-1 concentrations in the inner ear has been completed. We will now move onto sample testing.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
In this study, we are examining the efficacy of a potential early intervention therapeutic for noise induced hearing loss (NIHL). The generation of reactive oxygen species (ROS) by mitochondria is an underlying mechanism of noise-induced damage to tissues in the inner ear that leads to noise-induced loss of hearing sensitivity. Our results demonstrate that inhibition of the mitochondrial fission process with a small molecule inhibitor significantly attenuates NIHL and reduces damage to the tissues of the inner ear. Additionally, this study is utilizing a localized application of the therapeutic molecule to the outer ear canal that limits systemic exposure and allows for a higher local concentrations in the cochlea. The observed reduction in noise-induced loss of hearing sensitivity and increased auditory hair cell survival with outer ear canal application of mdivi-1 demonstrates the efficacy of this route of application and has potential relevance for the administration of other therapeutic agents for NIHL.

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

A delay in the start of Major Task 2 occurred due to a change in the size of the sound exposure booth for our study to a smaller unit was required by our Fire Marshall. A request for this change, which included a budget change due to the lower cost, received approval on 11/19/2015. Unfortunately, we discovered at the end of December that the chosen vendor, IAC Acoustics, had temporarily suspended booth production due to new ownership. The company that acquired IAC's assets completed construction of our booth at the end of August and installation was completed on 09/20/2016. Due to this delay, we are behind the dates of the approved SOW schedule, but we are making progress on the studies of Major Task 2 utilizing the OHRC shared sound exposure booth, a solution that allows progress to be made, if not at an optimal rate. We have also initiated our studies of Major Task 4 (HPLC measurement of mdivi-1 levels in cochlea) in an attempt to make up for some of the lost time.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

Publications, conference papers, and presentations

Nothing to report

Journal publications

Nothing to report

Books or other non-periodical, one-time publications

Nothing to report

Other publications, conference papers, and presentations

Nothing to report

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

1. Name: Alfred Nuttall
   Project Role: PI
   Nearest Person Month Worked: 2 Calendar months
   Contribution to Project: Dr. Nuttall supervised the equipment purchases, space renovation. calibration of the ABR/DPOAE system, and progress of the study experiments.

2. Name: Teresa Wilson
   Project Role: Co-I
   Nearest Person Month Worked: 9 Calendar months
   Contribution to Project: Dr. Wilson assisted with account set-up, equipment purchases, and space renovation in coordination with Dr. Nuttall and the Department Manager. She also worked on the experimental projects of Major Task 1, Major Task 2 and Major Task 4.

3. Name: Edward Porsov
   Project Role: Engineer
   Nearest Person Month Worked: 1 Calendar months
   Contribution to Project: Mr. Porsov completed the equipment requisitions/ordering and worked with the Fire Marshall to resolve equipment issues. He also completed the ABR/DPOAE system set-up and system calibration.

4. Name: Sarah Foster
   Project Role: Research Assistant
   Nearest Person Month Worked: 11 Calendar months
   Contribution to Project: Ms. Foster assisted with equipment ordering and clear-out of the sound exposure booth space. She also assisted with the set-up and calibration of the ABR/DPOAE system. She worked on the experimental projects of Major Task 1, Major Task 2 and Major Task 4.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS: An updated Quad chart is attached to this report

Controlling Mitochondrial Dynamics to Mitigate Noise-Induced Hearing Loss

MR141227
W81XWH-1-0560

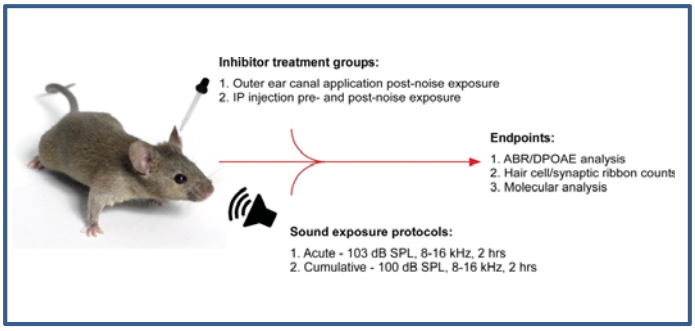
PI: Nuttall, Alfred L. **Org:** Oregon Health & Science University **Award Amount:** $1,233,279

Study Aims

• *Specific Aim 1*: Determine the optimal dose and dosing regimen for outer ear canal application and intraperitoneal injection of mdivi-1 for mitigation of NIHL resulting from a single steady-state noise exposure.
•*Specific Aim 2*: Determine whether inhibition of mitochondrial fission can protect against the adverse mitochondrial-based cellular consequences of loud sound exposure.
• *Specific Aim 3*: Determine whether outer ear canal administration of mdivi-1 will prevent the cumulative effects of multiple steady-state loud sound exposures over an extended time period.

Approach

The well-characterized CBA/CaJ mouse model will be used to determine the optimal dose and timing of inhibitor administration in the prevention of NIHL. Functional tests for hearing sensitivity will include auditory brainstem responses and distortion product otoacoustic emissions measurements. Molecular and cellular endpoints will be examined for the mitochondrial-dependent mechanisms leading to tissue damage, and the inhibitor's ability to attenuate these resulting in reduced NIHL.



Accomplishment: ACURO approval, ABR/DPOAE system set-up and sound exposure booth installation completed. Experimental studies of Task 2 (outer ear application of mdivi-1) and Task 4 (HPLC analysis) are in progress.

Timeline and Cost

Activities CY	15	16	17	18
Regulatory approvals and equipment ordering/set-up				
Specific Aim 1: Single steady-state induced NIHL, mdivi-1 dose and timing optimization				
Specific Aim 2: Etiology of mdivi-1 protection against NIHL				
Specific Aim 3: Cumulative steady-state induced NIHL, dose and timing optimization				
Estimated Budget ($K)		**$479**	**$371**	**$383**

Updated: 10/13/2016

Goals/Milestones
CY15 Goals – Obtain regulatory approvals and equipment ordering
■ ACURO approval
◘ Equipment ordering and set-up: ABR/DPOAE system has been set-up and the sound exposure booth installed.
CY16 Goal – Mdivi-1 protection against acute loud sound exposure
◘ Outer ear canal inhibitor application: Task 2 is in progress.
☐ IP injection of inhibitor
◘ HPLC quantification of cochlea inhibitor concentration is underway
CY17 Goals – Molecular and cellular basis of mdivi-1 protection
☐ Examine markers of mitochondrial health
☐ Quantify mitochondrial ROS production following noise exposure
CY18 Goal – Mdivi-1 protection against multiple loud sound exposures
☐ Outer ear canal inhibitor application after each of
Comments/Challenges/Issues/Concerns
We are behind the SOW schedule, but will be able to make up significant time with the installation of the sound exposure booth.
Budget Expenditure to Date
Projected Expenditure: $405,000 (Total costs)
Actual Expenditure: $384,964 (Total costs).